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Isolation of bacteriophage Kp14 and its combination of various antibiotics against *Klebsiella pneumoniae* strain

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ABSTRACT: *Klebsiella pneumoniae* is a critical pathogen frequently encountered in healthcare environments and is associated with a range of severe infections, including urinary tract infections, septicemia, meningitis, pneumonia, and surgical site infections. Furthermore, this bacterium can impact healthy, young individuals globally, rendering it a significant public health concern. Bacteriophages, viruses that specifically infect bacteria, present a promising alternative for the treatment of bacterial infections. The synergy between bacteriophages and antibiotics represents an effective therapeutic strategy, as it enhances antimicrobial efficacy and diminishes the likelihood of resistance development. This study assessed the in vitro activity of antibiotics, both independently and in conjunction with an isolated lytic bacteriophage, against a clinical strain of *K. pneumoniae*. A clinical *K. pneumoniae* strain was employed to isolate lytic bacteriophages. The lytic bacteriophage Kp14, isolated from lake water, produced clear plaques. The effects of combined treatments involving four antibiotics (ciprofloxacin, amikacin, meropenem, and ceftazidime) and the isolated bacteriophage were evaluated utilizing the time–kill assay. Synergistic activity was observed for ciprofloxacin and ceftazidime at both 1×MIC and 4×MIC, whereas synergism was observed for meropenem and amikacin at 4×MIC. Our findings demonstrate that bacteriophage therapy, when used in conjunction with conventional antibiotics, may serve as an effective alternative treatment for *K. pneumoniae* infections.

KEYWORDS: Antibiotic resistance; synergism; time-kill; bacteriophage therapy.

1. INTRODUCTION

Klebsiella pneumoniae is a Gram-negative nosocomial pathogen that colonises the mucous membranes and can cause infections such as purulent abscesses, septicemia, and meningitis. Individuals with compromised immune systems are especially vulnerable to infections caused by *K. pneumoniae*, which can affect various organs, including the urinary tract, liver and lungs [1].

In recent years, diseases caused by *K. pneumoniae* have increased all over the world and antibiotic resistance has also emerged due to widespread antibiotic use. Multidrug-resistant (MDR) *K. pneumoniae* is challenging to treat due to its high levels of intrinsic and adaptive resistance to nearly all antibiotics [2]. Intrinsic resistance often arises from inherent properties such as the presence of porins in the outer membrane. Acquired resistance occurs when bacteria that were once susceptible develop resistance through mechanisms such as acquiring plasmids or other resistance genes, or through biofilm formation [3,4]. In Europe, most *K. pneumoniae* isolates are resistant to at least one major antimicrobial drug class, including third-generation cephalosporins, aminoglycosides, carbapenems, or fluoroquinolones [5]. The formation of biofilms also significantly enhances antibiotic resistance in *K. pneumoniae*, increasing it by up to 1,000 times. The US Centers for Disease Control and Prevention (CDC) reported that about 50% of infections over the last decade were linked to MDR *K. pneumoniae* [6].

K. pneumoniae is among the top priority pathogens (*Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Enterobacter* spp., *Acinetobacter baumannii*) determined by the World Health Organisation [7]. The key strategies to combat antibiotic resistance include infection control, the discovery of new and more effective antibiotics or antimicrobials, judicious antibiotic use, regulation of their clinical and industrial use, global surveillance, and public education.

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Bacteriophage (phage) therapy has emerged as a promising alternative for treating infections caused by antibiotic-resistant strains that do not respond to conventional antibiotics [8]. Phages are viruses that specifically infect and destroy bacteria, demonstrating promising therapeutic potential [9]. One of the major advantages of phage therapy is its high host specificity, allowing phages to selectively target particular bacterial species without harming human cells or the commensal microbiota [4].

Veterinary and clinical phage therapy was first introduced by Felix d'Hérelle (1873–1949) in 1921 [10]. Phage therapy has not yet been approved as a standard treatment by the U.S. Food and Drug Administration or the European Medicines Agency. There are uncertainties regarding the efficacy, availability, safety, and quality of phage therapy. However, some clinical trials for phage therapy have been approved on a case-by-case basis [11,12]. Phage therapy is often conducted under Article 37 of the Declaration of Helsinki, which allows for "compassionate care" when standard antimicrobial treatments are ineffective. Standard treatment protocols typically do not recommend antibiotic withdrawal, so phage therapy is often administered alongside antibiotics. Therefore, elucidating potential interactions among antimicrobial agents is essential, and the number of phage research centres is rapidly increasing in many countries worldwide [13].

Phage-antibiotic combinations have been proposed to prevent the emergence of phage resistance and to help combat antibiotic-resistant bacteria. Phage-antibiotic combinations can produce different results: synergy, additive effect or antagonism. Phage-antibiotic synergy (PAS) is the bactericidal effect of antimicrobial substances, where the combined effect is greater than the sum of their effects individually. PAS treatment has been shown to inhibit bacterial pathogens, prevent the development of resistant variants, and decrease biofilm formation [14].

In the present study, the isolated bacteriophage Kp14 was evaluated against 20 clinical *K. pneumoniae* isolates, while the synergistic antibacterial effects of the tested phage–antibiotic combinations were investigated against the *K. pneumoniae* host strain.

2. RESULTS

2.1. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on the *K. pneumoniae* host strain and the results are presented in Table 1. Minimum inhibitory concentration (MIC) values for the antibiotics against the quality control strain were within the Clinical and Laboratory Standards Institute (CLSI) accuracy range. The MIC results for *K. pneumoniae* isolate indicated that this clinical isolate was resistant to ceftazidime (CAZ) and susceptible to meropenem (MEM), amikacin (AMK) and ciprofloxacin (CIP).

Table 1. MICs of antibiotics against the *K. pneumoniae* host strain.

Strain	Antibiotics	MICs (mg/L)
K. pneumoniae host strain	CIP	0.25
	CAZ	32
	MEM	0.06
	AMK	4

CIP, ciprofloxacin; CAZ, ceftazidime; MEM, meropenem; AMK, amikacin
CLSI establishes the breakpoints that define susceptibility and resistance for *Enterobacteriaceae*: MEM at ≤1 mg/L and ≥4 mg/L,
for CIP at ≤ 1 mg/L and ≥4 mg/L, for CAZ at ≤ 4 mg/L and ≥16 mg/L, for AMK at ≤ 16 and ≥64 mg/L.

2.2. General characteristics of phage Kp14

Phage Kp14 was isolated from lake water in Istanbul, Turkiye. The phage formed clear plaques on its bacterial host (Figure 1). The lytic spectrum of phage Kp14 was evaluated against 20 clinical *K. pneumoniae* isolates using the spot assay method. Among the tested isolates, phage Kp14 exhibited confluent lysis (+++) against three isolates (15%), semi-confluent lysis (++) against two isolates (10%), and individual plaque formation (+) against one isolate (5%). No lytic activity was observed against 14 isolates (70%) (Table 2).
Multiplicity of infection (MOI) is defined as the ratio of phage particles to host cells that yields maximum phage production. The optimal MOI of the phage was 1 (Figure 2).

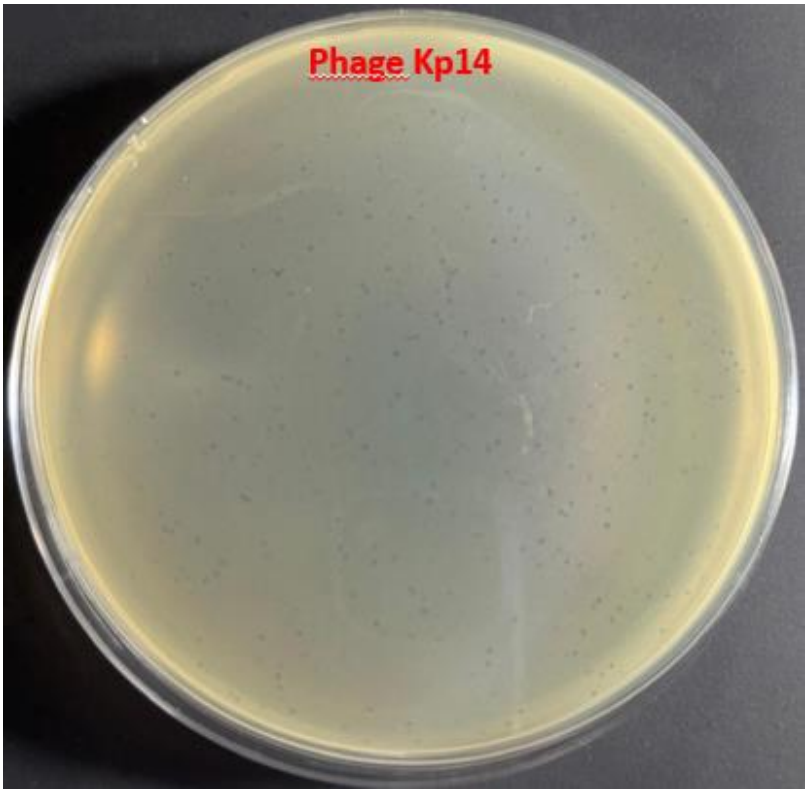


Figure 1. Plaque morphology of phage Kp14 on its host bacterium using the double-layer agar method

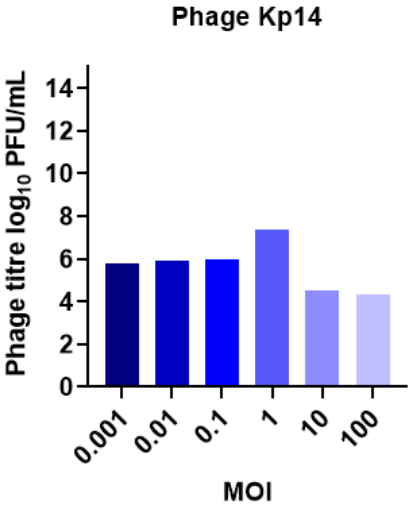


Figure 2. MOI of phage Kp14

Table 2. Spot test results of phage Kp14 against *K. pneumoniae* isolates

Phage	Isolates	Results of spot test (%)			
		+++	++	+	R
Kp14	*K. pneumoniae* clinical isolates (n=20)	3 (15%)	2 (10%)	1 (5%)	14 (70%)

Confluent lysis (+++), semi-confluent lysis (++), individual plaques (+) and no lysis (R).

2.3. Time-kill assay

To assess the synergistic effects of phage Kp14 combined with antibiotics against the host *K. pneumoniae* isolate, the antimicrobial activities of antibiotics alone, phage alone, and their combinations were evaluated.

The time-kill assay demonstrated bactericidal activity, defined as a ≥3 $\log_{10}$ CFU/mL reduction in bacterial count within 24 h. Most phage–antibiotic combinations exhibited bactericidal activity at 1×MIC and 4×MIC concentrations (Figure 3). The MEM–phage Kp14 and AMK–phage Kp14 combinations demonstrated additive effects at 1×MIC, whereas synergistic interactions were observed for all combinations at 4×MIC concentrations. In addition, the CAZ–phage Kp14 and CIP–phage Kp14 combinations exhibited synergistic activity at both 1×MIC and 4×MIC concentrations (Table 3).

Table 3. Synergistic and bactericidal effects of phage-antibiotic combinations against the *K. pneumoniae* host strain

COMBINATION EFFECTS															
CAZ-phage Kp14				MEM-phage Kp14				AMK-phage Kp14				CIP-phage Kp14			
1×MIC		4×MIC		1×MIC		4×MIC		1×MIC		4×MIC		1×MIC		4×MIC	
S/A	Bct	S/A	Bct	S/A	Bct	S/A	Bct	S/A	Bct	S/A	Bct	S/A	Bct	S/A	Bct
S	+	S	+	A	-	S	+	A	-	S	+	S	+	S	+

CAZ, ceftazidime; MEM, meropenem; AMK, amikacin; CIP, ciprofloxacin; Bct, bactericidal; S/A, synergistic/additive

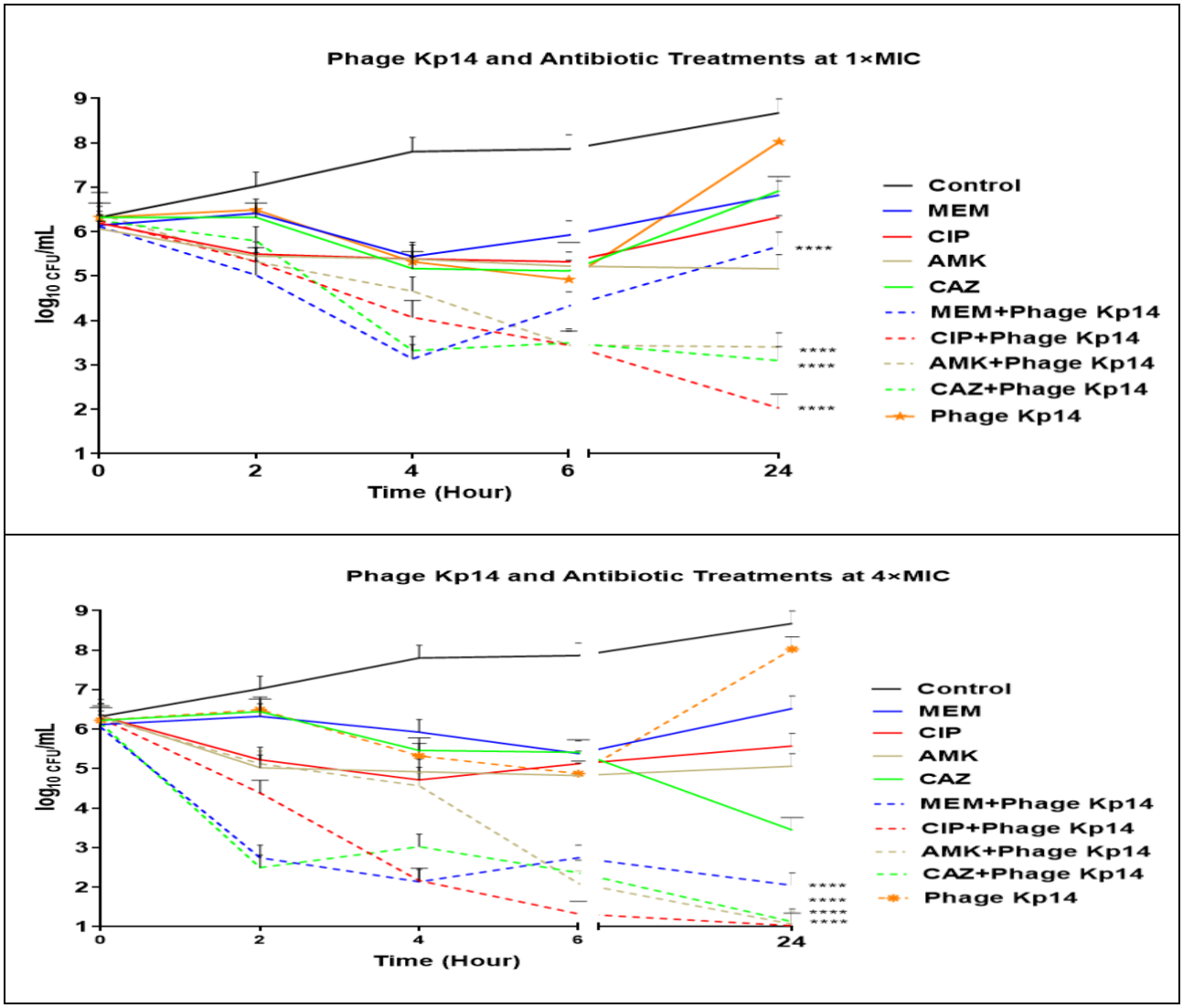


Figure 3. In vitro activities of MEM, CIP, CAZ, and AMK at 1× MIC and 4× MIC, and phage Kp14 alone and in combination against the *K. pneumoniae* host strain. The x-axis represents the duration of killing, whereas the y-axis indicates the logarithmic survival of the *K. pneumoniae* host strain (****$p < 0.0001$).

3. DISCUSSION

K. pneumoniae is a clinically significant Gram-negative bacterium associated with healthcare-associated infections and public health challenges. Natural or acquired antibiotic resistance, as well as the ability to produce biofilm, significantly increases resistance in *K. pneumoniae*, thereby complicating treatment and leading to high mortality rates. Given the need for new therapeutic strategies, adjuvant antibiotic therapy has been investigated for years [15]. Infections caused by ESKAPE pathogens, including *Enterobacter* species, *S. aureus*, *P. aeruginosa*, and *K. pneumoniae*, have the potential to be treated with phage therapy [16-19].

Although lytic bacteriophages have been proposed as an alternative therapeutic approach, it is crucial to first characterise the specific bacteriophages and their expanded infectivity to optimise efficacy against the target organism [20]. Phage host range is determined by complex interactions, and most phages can infect only a specific subset of strains of a particular bacterial species. In contrast, some phages exhibit broader host ranges, enabling infection of different bacterial species [21] or even taxonomically distinct genera [22]. Many environmental and opportunistic bacteria have an extracellular capsule. Capsules protect bacteria from the immune systems of multicellular organisms and are considered significant virulence factors. To counteract the bacterial capsule, some bacteriophages encode enzymes known as depolymerases that cleave specific oligosaccharide bonds [23,24]. While many *Klebsiella* phages have a narrow host range due to specific

interactions with various capsular polysaccharides, this feature provides significant therapeutic advantages. High host specificity reduces off-target effects on the commensal microbiota, aligning with personalised antimicrobial strategies. Research indicates that targeted isolation methods can identify phages specific to clinically significant *Klebsiella* serotypes, enabling the formulation of narrow-spectrum phage cocktails to enhance effectiveness and slow the emergence of resistance [25,26]. Studies have shown that many *Klebsiella* phages possess a narrow host range, typically limited to specific capsular types; this highlights the challenge of identifying broadly effective therapeutic phages [25,27]. In the present study, we found that phage Kp14, isolated from lake water in Istanbul, Turkiye, produced small, clear plaques and exhibited confluent lysis against 15% of the 20 tested *K. pneumoniae* isolates.

The combination of phages and antibiotics has the potential to enhance overall efficacy. However, it is crucial to determine any possible synergistic interactions through empirical research [14]. In this study, we explored the efficacy of phage Kp14 in combination with CAZ, MEM, AMK, and CIP at 1×MIC and 4×MIC concentrations. The bacterial host was characterised as susceptible to MEM, CIP, and AMK and resistant to CAZ. Our study showed that the combination of phage Kp14 with MEM exhibited an additive effect at 1×MIC, whereas a synergistic effect was observed at 4×MIC. In contrast, the combination of phage Kp14 with CAZ demonstrated a synergistic effect at both 1× and 4×MIC concentrations, despite bacterial resistance. MEM and CAZ are both β-lactams that bind to penicillin-binding proteins in the bacterial cell wall, thereby inhibiting peptidoglycan synthesis. It is likely that combinations of β-lactams and bacteriophages may be more effective than other phage-antibiotic combinations [28,29]. This phenomenon can be explained by the PAS, whereby higher antibiotic concentrations increase bacterial stress, disrupt cell wall synthesis, and increase susceptibility to phage adsorption and replication. Subpopulations that survive lower antibiotic doses may remain resistant to phage infection, while higher concentrations may sensitise a larger portion of the population. Synergistic interactions between phages and β-lactam antibiotics have been observed in multidrug-resistant *K. pneumoniae*, showcasing the potential of combination therapies to enhance antibacterial efficacy and minimise the development of resistance [30].

The time-kill assay indicated that the combination of AMK at 4×MIC and phage Kp14 demonstrated significantly enhanced efficacy. AMK exerts bactericidal activity by disrupting bacterial protein synthesis and enhancing cellular respiration. Phage-induced bacterial membrane disintegration may reduce permeability barriers and enhance amikacin's entry into its target, allowing for synergistic interactions that produce potent bactericidal effects against these pathogens. Previously, phage-AMK combinations have demonstrated synergistic effects, even at sub-inhibitory concentrations of antibiotics, effectively suppressing bacterial growth and preventing regrowth over extended periods [31]. In the present study, however, a synergistic effect was observed only at the 4×MIC concentration, indicating that the degree of synergy may depend on antibiotic dosage, phage–host interactions, or strain-specific characteristics.

CIP is a fluoroquinolone antibiotic that kills bacteria by inhibiting DNA gyrase and topoisomerase IV, thereby blocking DNA replication and causing lethal damage in susceptible organisms. Phage Kp14-CIP interactions may enhance the synergy between phages and antibiotics. Furthermore, research suggests that combining phages with CIP can enhance bacterial eradication in polymicrobial communities. This indicates that the complementary actions of CIP and phages may yield greater antibacterial effects than using either treatment alone. In the present study, the CIP–phage Kp14 combination demonstrated synergistic activity at both 1× and 4×MIC concentrations. Similar synergistic interactions have been documented in MDR *K. pneumoniae* and other Gram-negative pathogens. CIP–phage combination therapy shows promise against bacterial infections [32].

Despite these promising findings, this study has several limitations. First, only a single phage was evaluated, and the number of clinical isolates tested was limited. The antibacterial efficacy of phage-antibiotic combinations was evaluated only under in vitro conditions and at selected antibiotic concentrations against a single host strain. Therefore, the observed synergistic interactions may not fully reflect in vivo conditions. Furthermore, genomic characterization of phage Kp14 was not performed in this study but will be investigated in future studies to better assess its therapeutic safety and potential. Future studies involving larger isolate collections, genomic characterization, and animal infection models are needed to better define the therapeutic potential and clinical applicability of phage-antibiotic combination therapy.

4. CONCLUSION

The present study examined the antibacterial effects of phage Kp14 in combination with various antibiotics against a clinical *K. pneumoniae* isolate. The observed synergistic interactions indicate the potential

of phage–antibiotic combinations against antibiotic-resistant *K. pneumoniae*. However, further studies using a broader range of clinical isolates are needed to confirm these findings, elucidate the mechanisms underlying these interactions, and evaluate their efficacy under in vivo conditions.

5. MATERIALS AND METHODS

5.1. Bacterial strain

In this study, a clinical isolate of *K. pneumoniae* was used for bacteriophage isolation. The isolate was obtained from the Culture Collection of the Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, Istanbul University, Turkiye. Bacterial cultures were grown in Luria–Bertani (LB) broth at 37 °C for 24 h. Phage propagation was carried out using LB broth, LB agar (1.5% w/v agar), and LB soft agar (0.7% w/v agar).

5.2. Isolation and purification of bacteriophage

Phage Kp14 was isolated under sterile conditions from lake water in Istanbul, Turkiye. Phage isolation, enrichment, and purification were performed as previously described [20]. Briefly, 10 mL of lake water was centrifuged at 4 °C at 5000 × g for 10 minutes to remove cell debris. The supernatant was filtered through a sterile 0.22 µm pore filter and transferred to a sterile tube. To verify the presence of phage in the filtrate, the filtrate was applied to the clinical *K. pneumoniae* strain in top agar and incubated overnight at 37 °C. The bacterial strain exhibiting a lytic zone was selected as an indicator bacterium for ongoing studies.

Phage purification was performed using the double-layer agar method [33]. Serial dilutions of the isolated phage were prepared in Saline Magnesium (SM) buffer. 200 µL of an overnight indicator bacterial culture was mixed with 200 µL of each dilution in 4 mL of soft agar (0.7%, w/v), and the mixture was poured onto LB agar plates (1.5%, w/v). The plates were incubated at 37 °C overnight. The following day, individual plaques were carefully selected and extracted from the plates based on their morphological and size characteristics. Selected phage plaques were placed in separate sterile tubes, each containing 200 µL of fresh LB broth inoculated with overnight indicator bacteria. The phage was harvested by centrifugation, and the supernatant was filtered through a sterile 0.22 µm filter. The procedure was repeated approximately five times. The lytic activity of phage Kp14 against twenty bacterial strains was evaluated using the spot assay method, as previously described [20].

5.3. MOI

MOI represents the ratio of infecting phages to bacterial cells, and the MOI yielding the highest phage titer was considered optimal. To determine the optimal MOI, exponentially growing host bacteria and isolated phage were mixed at various MOIs (0.01–100) and incubated at 37 °C for 4 h. Phage titres (PFU/mL) were then determined using the double-layer agar method to identify the optimal MOI [34].

5.4. Antimicrobial susceptibility testing

MICs of antibiotics were determined by broth microdilution, following the CLSI guidelines, against the *K. pneumoniae* host strain [35]. Serial two-fold dilutions of CAZ, MEM, AMK and CIP were prepared in cation-adjusted Mueller-Hinton broth (CAMHB). A 0.5 McFarland standard was used to measure the turbidity of bacterial cultures. The inoculum was then diluted in CAMHB to obtain approximately 5×10^5 CFU/mL. Each trial was conducted three times.

5.5. Time-kill assay

To assess the bactericidal effects of the phage-antibiotic combination against the host bacterium *K. pneumoniae*, time-dependent killing assays were performed. Phage (MOI: 1), bacteria (10^8 CFU/mL), and bacteria with antibiotics (1×MIC, 4×MIC) were monitored simultaneously in tubes. After incubation, bacterial counts were determined at 0, 2, 4, 6, and 24 h by colony counting. The time–kill curve analysis was performed based on the NCCLS criteria [36]. Synergy was defined as a reduction of ≥2 log10 CFU/mL in bacterial counts at 24 h compared to the most effective single agent. An additive effect was defined as a <2 log10 CFU/mL difference compared with the most active single agent. Antagonism was defined as a ≥2 log10 CFU/mL increase in bacterial counts compared with the most active single agent. Bactericidal activity was defined by a reduction of more than 3-log10 CFU/mL from the initial level. Each experiment was performed three times.

5.6. Statistical analysis

All studies were carried out in three separate trials. Two-way ANOVA with Tukey's multiple comparisons test was used to assess differences between the groups where antibiotics were administered alone or in combination with phage Kp14. A p-value < 0.0001 (****) was considered statistically significant.

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